Label-free Third Harmonic Generation Imaging of Lipid Droplets in Live Filamentous Fungi

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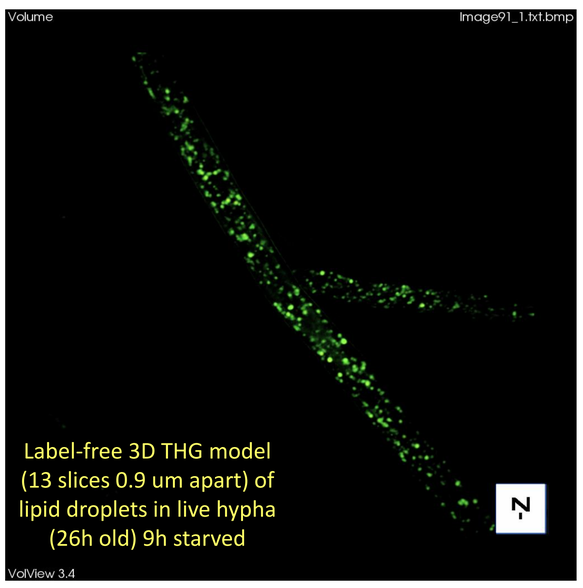
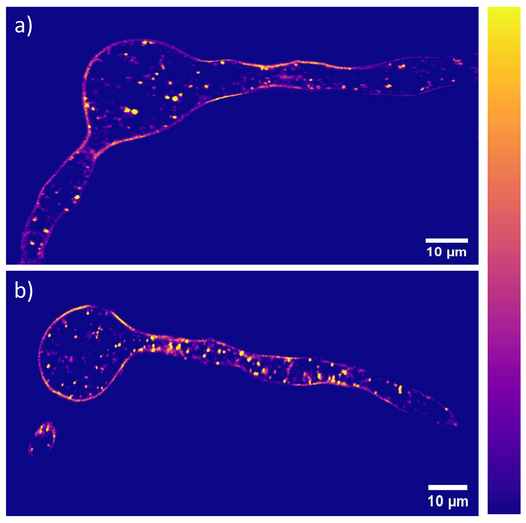
Introduction

Third harmonic generation (THG) microscopy is a label-free nonlinear imaging technique. THG mostly occurs at interfaces where the change of refractive index is steep, like water-lipid structures. Here, we present *in vivo* and label-free THG imaging of individual hyphae of the oleaginous filamentous fungus *Phycomyces blakesleeanus*, where lipid droplets (LDs) are the main source of contrast. The LDs quantification from THG images was performed by two image analysis techniques:

1) Image Correlation Spectroscopy (ICS)

2) Particle Size Analysis (PSA) - software particle counting





Label-free THG images of lipid droplets in live hyphae, a) control (28h old; $P_{imaging}$=24mW) and b) 5h starved (26h old; $P_{imaging}$=26mw) hyphae

Conclusions

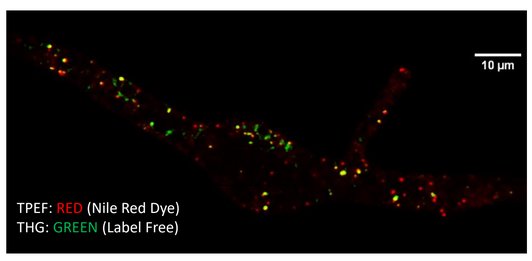
The THG method, *in vivo* and label-free, accurately and reliably, over time, detected changes in the localization, total number, and size of LDs in hyphae of the filamentous fungus *Phycomyces blakesleeanus.*

ICS recognized lipid droplets of smaller diameter and consistently detected slightly larger LDs numbers in the older hyphae, compared to PSA.

Experiment

- For THG imaging of label-free hyphae, 1040 nm, 200 fs pulses from Yb KGW laser was used
- Live 23+ old hyphae were observed between two 170µm thick cover glasses in specially designed sample holder

Colocalization of LDs imaged by TPEF and THG

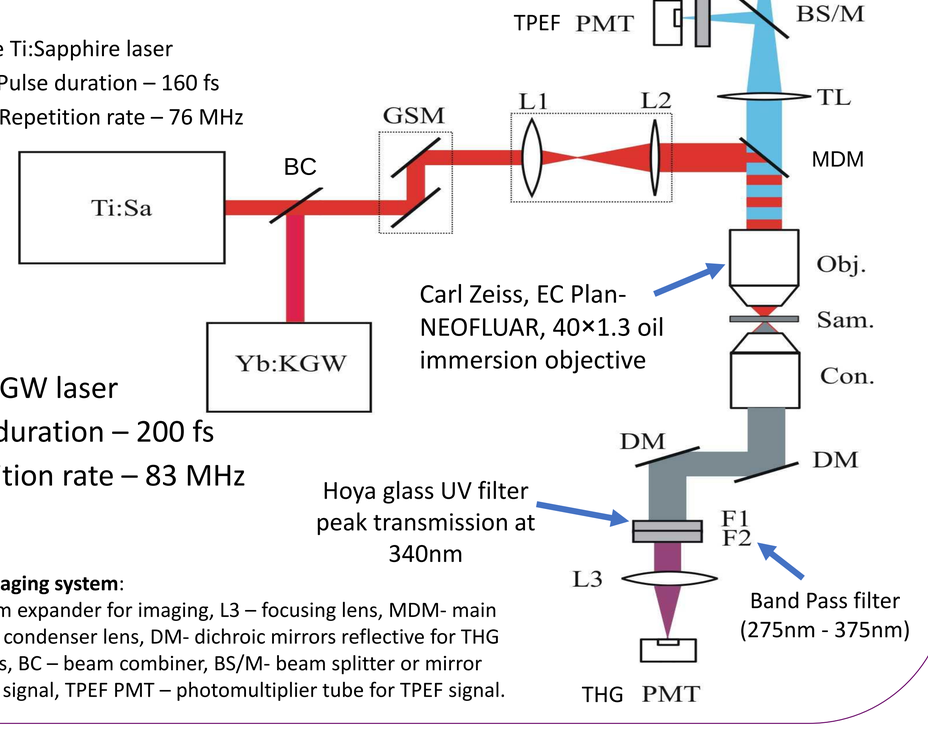


For *In Vivo TPEF* imaging of hyphae labeled by Nile Red dye we used same laser

NLSM setup

The Ti:Sapphire laser
- Pulse duration – 160 fs
- Repetition rate – 76 MHz

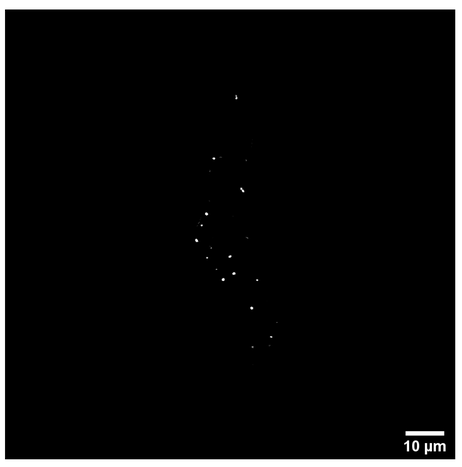
The Yb KGW laser
- Pulse duration – 200 fs
- Repetition rate – 83 MHz



Schematic of the imaging system:
GSM- galvo scanning mirrors, L1 and L2 - lenses of 1:3.75 beam expander for imaging, L3 – focusing lens, MDM- main dichroic mirror (cut-off 700 nm), Sam.- sample, Con.- aspheric condenser lens, DM- dichroic mirrors reflective for THG (347nm) and transmitive for Yb laser (1040nm), TL- tube lens, BC – beam combiner, BS/M- beam splitter or mirror toggle, Cam.- camera, THG PMT- photomultiplier tube for THG signal, TPEF PMT – photomultiplier tube for TPEF signal.

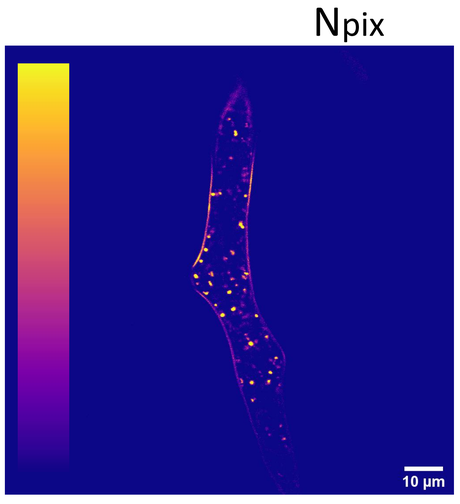
THG images analysis and quantification of lipid droplets by two methods

Image Correlation Spectroscopy (ICS)



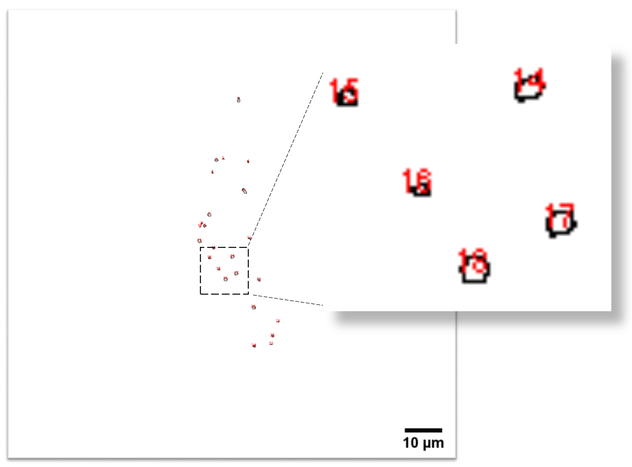
c) The image from a) after 20x background subtractions

Npix



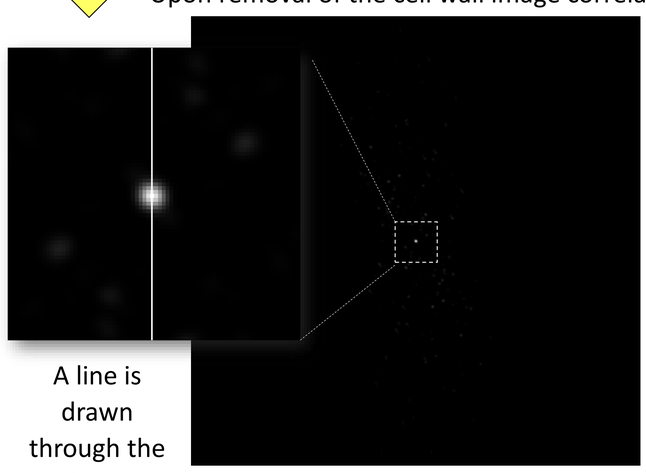
a) The unprocessed THG image of 29 hours old hypha (6.5h starved)

Particles Size Analysis (PSA)



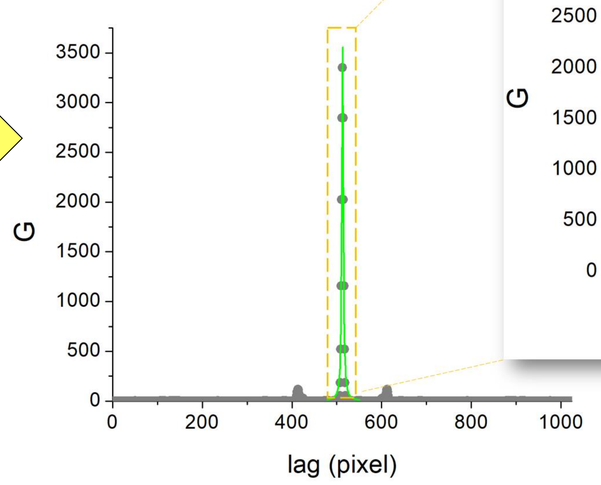
b) 8-bit mask after Particle size analysis

Upon removal of the cell wall image correlation procedure is performed in Image J

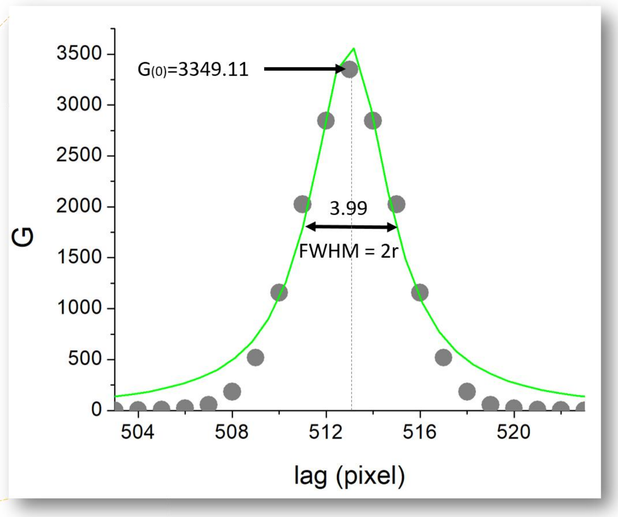


A line is drawn through the brightest pixel in the center of the resulting image

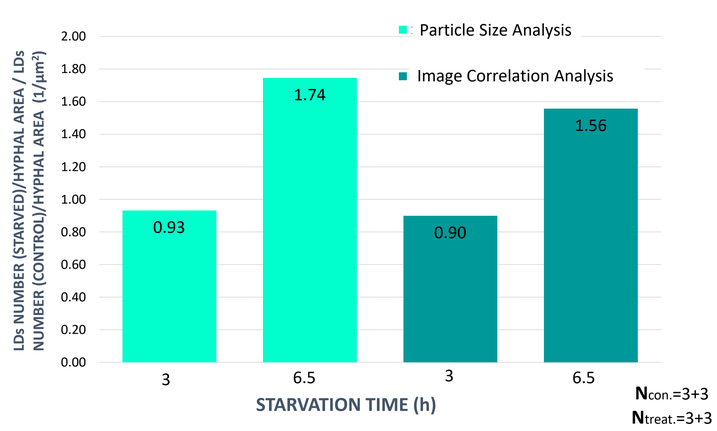
d) As the result, one obtains a correlation image from which the G curve is extracted as an intensity profile through the center of the image.



e) The autocorrelation curve - G curve (grey circles) was fitted by a Lorentzian (green line) in order to extract the FWHM value.



$$N_{LD} = \frac{N_{pix} \cdot N_{pix}}{r^2 \pi \cdot G(0)}$$ r - radius



Control/Treatment	Image Correlation Analysis: N of LDs / hyphae area (1/µm2) x 10^{-3} (mean ± SD)	Image Correlation Analysis: Mean LDs diameter (µm)	Particles Size Analysis: N of LDs / hyphae area (1/µm2) x 10^{-3} (mean ± SD)	Particles Size Analysis: Mean LDs diameter (µm)
Control	15 ± 0.1	0.6	16 ± 4	0.7
3h Nitrogen-starved cells	14 ± 5	0.6	15 ± 10	0.8
Control	16 ± 8	0.5	13 ± 5	0.8
6.5h Nitrogen-starved cells	24 ± 5	0.4	22 ± 6	0.8

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